Correlation between chronological and physiological age of males from their multivariate urinary endogenous steroid profile and prostatic carcinoma-induced deviation

Elsevier Editorial System(tm) for Steroids
Manuscript Draft

Manuscript Number: STEROIDS-D-18-00128R2

Title: Correlation between chronological and physiological age of males from their multivariate urinary endogenous steroid profile and prostatic carcinoma-induced deviation

Article Type: Regular Article

Keywords: Urinary steroid profile (USP); physiological age; GC-MS; Kernel-PLS (K-PLS) regression; prostatic carcinoma (PCa)

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Abstract: The biosynthesis of endogenous androgenic anabolic steroids (EAAS) in males varies with age. Knowledge of the general urinary EAAS profile's dependence from aging - not reported up to now - may represents a prerequisite for its exploitation in the screening and diagnostic support for several pathologies. Extended urinary EAAS profiles were obtained from healthy and pathological individuals, using a GC-MS method which was fully validated by a stepwise, analyst-independent scheme. Seventeen EAAS and five of their concentration ratios were determined and investigated using multivariate statistical methods. A regression model based on Kernel partial least squares algorithm was built to correlate the chronological age of healthy male individuals with their "physiological age" as determined from their urinary EAAS profile. Strong correlation (R2=0.75; slope=0.747) and good prediction ability of the real chronological age was inferred from EAAS data. In contrast, patients with recent diagnosis (not pharmacologically treated) of prostatic carcinoma (PCa) exhibited a comprehensive EAAS profile with strong negative deviation from the model, corresponding a younger predicted age. This result is possibly related to the activation of anomalous steroid biosynthesis induced from PCa. Over a restricted 60-80 years-old population, PLS-discriminant analysis (DA) was used to distinguish healthy subjects from patients with untreated PCa. PLS-DA yielded excellent discrimination (sensitivity and specificity > 90%) between healthy and pathological individuals. This proof-of-concept study provides a preliminary evaluation of multivariate DA on wide EAAS profiles as a screening method to distinguish PCa from non-pathological conditions, overcoming the potentially interfering effect of ageing.

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Torino, August 10th, 2018

To the Editor-in-Chief of "Steroids", Prof. John A. Cidlowski

Subject: Submission of revised manuscript Ms. Ref. No.: STEROIDS-D-18-00128

Dear Prof. Cidlowski,
This letter accompanies the further **revision** (minor issues) of the manuscript STEROIDS-D-18-00128 entitled **"Correlation between chronological and physiological age of males from their multivariate urinary endogenous steroid profile and prostatic carcinoma-induced deviation"**.
The authors are:
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Editor:
1) The present cover letter is loaded as a Word file (the previous one was a pdf file)
2) Figure 2 has been improved and replaced. The new TIFF file has adequate resolution.
3) The "Highlight" file has been revised and modified. The new file has been uploaded.

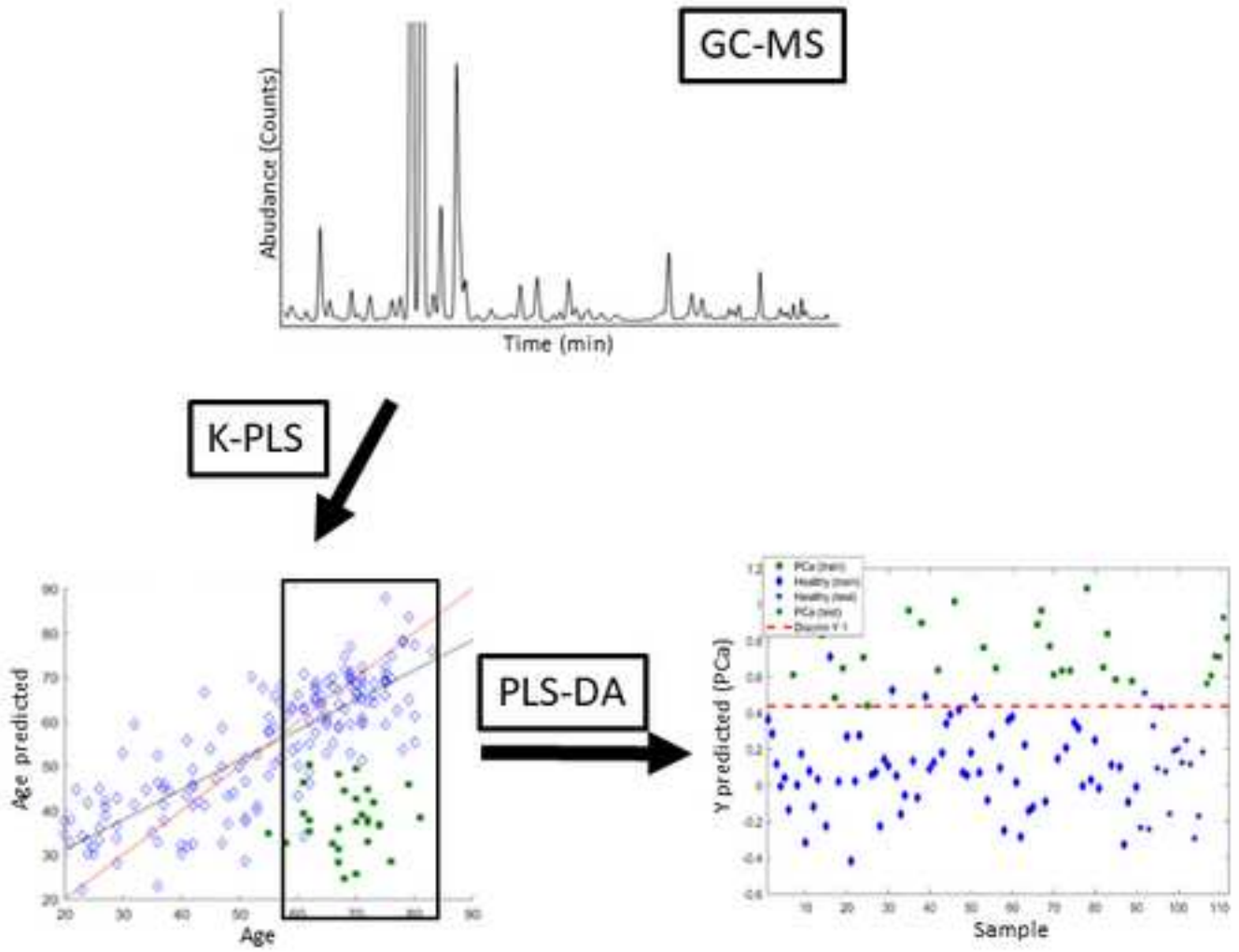
Reviewer #1:
1)-6) All the minor changes suggested by the Reviewer has been included in the revised manuscript.

Best regards.

Yours faithfully,

Marco Vincenti

Revised Graphical Abstract



***Highlights (for review)**

- An efficient GC-MS method is validated for a panel of 18 urinary steroids
- A Kernel-PLS algorithm investigates the age dependence of the steroid panel
- The age-regression model does not hold for subjects affected by prostate carcinoma
- PLS-DA on steroid panel discriminates healthy from pathological individuals

CORRELATION BETWEEN CHRONOLOGICAL AND PHYSIOLOGICAL AGE OF MALES FROM THEIR MULTIVARIATE URINARY ENDOGENOUS STEROID PROFILE AND PROSTATIC CARCINOMA-INDUCED DEVIATION

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keywords: Urinary steroid profile (USP), physiological age, GC-MS, Kernel-PLS (K-PLS) regression, prostatic carcinoma (PCa),

List of abbreviations:

5α-adiol: 5α-androstan-3α,17β-diol

5β-adiol: 5β-androstan-3α,17β-diol

A: androsterone

ANOVA: analysis of variance

AUC: area under the curve

CV%: coefficient of variation

CV: cross validation

DA: discriminant analysis

DHEA: dehydroepiandrosterone

DHT: dihydrotestosterone

DoE: design of experiment

E: epitestosterone

EAAS: endogenous androgenic anabolic steroids

Etio: etiocholanolone

GA: genetic algorithms

GC-MS gas chromatography-mass spectrometry

GnRH; gonadotropin-releasing hormone

LOD: limit of detection

LOQ: limit of quantification

MSTFA: N-Methyl-N-(trimethylsilyl)trifluoroacetamide

PCa: prostatic carcinoma

PLS: partial least squares

PSA: prostate specific antigene

RMSECV: root-mean-square error in cross-validation

RMSEP: root-mean-square error in prediction

ROC: receiver operating characteristic

SG: specific gravity

SPE: solid phase extraction

T: testosterone

TBME: methyl tert-butyl ether

TRUS: trans-rectal ultrasound guided

USP: urinary steroid profile

No "Human Genes" is discussed in the paper.

ABSTRACT

The biosynthesis of endogenous androgenic anabolic steroids (EAAS) in males varies with age. Knowledge of the general urinary EAAS profile's dependence from aging - not reported up to now - may represents a prerequisite for its exploitation in the screening and diagnostic support for several pathologies. Extended urinary EAAS profiles were obtained from healthy and pathological individuals, using a GC-MS method which was fully validated by a stepwise, analyst-independent scheme. Seventeen EAAS and five of their concentration ratios were determined and investigated using multivariate statistical methods. A regression model based on Kernel partial least squares algorithm was built to correlate the chronological age of healthy male individuals with their "physiological age" as determined from their urinary EAAS profile. Strong correlation (R^2=0.75; slope=0.747) and good prediction ability of the real chronological age was inferred from EAAS data. In contrast, patients with recent diagnosis (not pharmacologically treated) of prostatic carcinoma (PCa) exhibited a comprehensive EAAS profile with strong negative deviation from the model, corresponding a younger predicted age. This result is possibly related to the activation of anomalous steroid biosynthesis induced from PCa. Over a restricted 60-80 years-old population, PLS-discriminant analysis (DA) was used to distinguish healthy subjects from patients with untreated PCa. PLS-DA yielded excellent discrimination (sensitivity and specificity > 90%) between healthy and pathological individuals. This proof-of-concept study provides a preliminary evaluation of multivariate DA on wide EAAS profiles as a screening method to distinguish PCa from non-pathological conditions, overcoming the potentially interfering effect of ageing.

1. INTRODUCTION

Sex hormones synthesis is regulated by the pulsating release of the hypothalamic gonadotropin-releasing hormone (GnRH) [1]. Testosterone (T) and epitestosterone (E) are produced inside the Leydig cells and converted in peripheral tissues to dihydrotestosterone (DHT), which accounts for most of T biological action [2]. Adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione are produced in the so-called *zona reticulata* of the adrenal cortex [3]. T undergoes extensive metabolic transformation before being excreted. In urine, the most abundant phase I metabolites of T are DHT, 5α-androstan-3α,17β-diol (5α-adiol), 5β-androstan-3α,17β-diol (5β-adiol), androsterone (A) and etiocholanolone (Etio) [4], together with dehydroepiandrosterone (DHEA), the testosterone precursor. In particular, high concentrations of A and Etio are detected [5]. Many other minor metabolites complete the urinary steroid profile (USP) [6–10].

One of the main factors for the USP inter-individual variability lies in the polymorphism of an enzyme belonging to the 2B subfamily of the (UDP)-glucuronosyl transferases UGT's subfamily 2B, the UGT2B17, principal responsible of the androgens glucuronidation [11]. The frequency of the UGT2B17 *(del/del)* genotype varies widely among ethnic backgrounds, showing higher frequency in East Asian population [12]. It is associated with variation in urine testosterone [13], with consequent bimodal distribution of its excretion as well as large differences in androgen excretion between Asians and Caucasians [11]. Beside the inter-individual variability associated with genetic polymorphism, the specific USP of each individual may be altered by a variety of causes, including the intake of interfering drugs and the onset of pathological conditions, making its definition a potential diagnostic tool of interest. T and DHT are significant for the development of sexual characters [14] whereas a significant T drop is associated with ageing. In the early seventies, several authors reported an age-associated decline of serum testosterone levels from the fourth/fifth decade of life [15,16]. Analogous age-dependence was observed for the serum levels of DHEA and its sulphated metabolite, while DHT is not apparently modified in elderly men [15]. Besides T and

few other precursors, there is a lack of scientific literature about the evolution of other excreted endogenous androgenic steroids and T metabolites with ageing, partly because their concentration depends on the circadian rhythm and other scarcely predictable factors [14–16]. On the other hand, some of their concentration ratios, i.e. T/E, A/T, A/Etio, 5α-adiol/5β-adiol and 5α-adiol/E, proved to be more stable and are consistently used in doping control [10,17]. The same ratios could be possibly included among the variables monitored in the USP models, in order to verify whether these urinary steroid concentration ratios used in the steroid profile of professional athletes to test illicit administration of anabolic steroids (called the "steroidal module of the athlete biological passport") may play a role also in the detection of the metabolic changes related with different causes, for example aging or prostatic malignancies, as in the present study.

Recently, our [18] and other research groups [19,20] proposed to interpret the results of an urinary endogenous steroids panel using the methods of multivariate statistical analysis, with the purpose to ascertain the intake of androgenic drugs [21,22] or even the deceitful substitution of urine samples in anti-doping controls [18]. The possible application to extended USP of similar multivariate statistical methods aimed to single out pathologies that modify the sexual hormones balance, among which prostatic carcinoma (PCa) [23], is interfered by the large inter-individual variability of steroid profiles due to genetic polymorphism and the general factors that modify them, including ageing. At least the latter variability factor can be tentatively rationalized by studying the characteristic changes induced by ageing on the steroid profile. Modelling the aging effect on the USP general variance may allow further inference on a general population of healthy and pathological subjects. On the other hand, the incidence of PCa generally increases with increasing age, with the largest percentage of diagnosed cases found in men 65-74 years old [23]. Since the correlation between PCa and increased levels of the most important androgens is well known [25], and considering the non-invasiveness of urine sampling, we decided to investigate the effectiveness of the USP for diagnosis of prostatic carcinoma using a simple GC-MS method combined with multivariate statistics..

A recent review by Bax et al. [24] provides an overview of the innovative approaches under study to improve the diagnosis of PCa. They can be grouped essentially as follows: (a) sensorial analysis (which relies on the mammalian sense of smell), (b) senso-instrumental analysis and (c) chemical analysis. The teams working with targeted chemical analysis found out that sarcosine, amino acids, and amines could represent powerful biomarkers for PCa. In particular, Derezinski et al. produced a Partial Least Squares – Discriminant Analysis (PLS-DA) model using a pool of amino-acids which yielded an accuracy exceeding 82 %. In the present study, we built a regression model that could predict the chronological age of healthy individuals according to their deconjugated steroidal profile, composed of 17 target steroids, and considered altogether as a multivariate dataset. This regression model explores the relationship existing between the physiological modification of the urinary steroid profile and the ageing of male individuals. A similar regression - purposely optimized - was preliminarily used to highlight systematic deviations from the model caused by ongoing pathologies that may affect the male hormone balance, in particular prostatic carcinoma (PCa). Individuals suffering from PCa, but still not under therapy, have been enrolled for this preliminary study. The prediction capability of this regression model was tested by discriminant analysis.

2. MATERIAL AND METHODS

2.1 CHEMICALS AND REAGENTS

All steroid standards were purchased as pure powders from Steraloids (Newport, RI, USA); DHT was provided by LGC Promochem (Milan, Italy). Methanol, methyl tert-butyl ether (TBME), ethyl acetate, 17α-methyltestosterone, dithioerythritol and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were provided by Sigma-Aldrich (Milan, Italy). β-glucuronidase from *Escherichia Coli*

was purchased from Roche Life Science (Indianapolis, IN, USA). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA).

Standards solutions were prepared in methanol at the concentration of 1 mg/mL. Then, three working solution mixtures were prepared by dilution. Two internal standards were used: testosterone-d_3 served for the quantification of all the target analytes, with the exception of A and Etio which were quantified by 17α-methyltestosterone. The list of the studied steroids, together with details about each working solution are reported in Table 1. All standard solutions were stored at −10°C until used.

2.2 EXPERIMENTAL DESIGN

A GC-MS method for the detection of 8 EAAS was validated in a previous study [18]. In the present study, a faster sample pre-treatment was optimized by means of design of experiment (DoE) [26], with the main objective of removing the SPE extraction step, following the experience of others research groups on similar ranges of target analytes [5,6,10].

Three critical factors were identified, namely (i) the optimal volume of β-glucuronidase enzyme used in the deconjugation step, within the experimental domain 50-100 µL, (ii) the solvent used for the extraction of the de-conjugated analytes (pentane and TBME were tested) (iii) the reaction time (30 to 50 minutes) allowed for the derivatization of the target analytes by MSTFA. A two levels full factorial DoE was employed plus one central point, giving a total of 9 experiments, performed in triplicate for a total number of 27 experiments (Supplemental Material – Table 1). The central point conditions were set as the middle values of quantitative parameters, together with a 1:1 (v/v) pentane - TBME mixture for the extraction. The testing solutions contained 125 ng/mL of MIX A analytes and 2250 ng/mL of MIX B analytes (see Table 1). MIX C was also added to the solution at the concentration of 125 ng/mL in order to test the enzymatic activity. The software R-3.4.2 was employed to perform the model computation [27,28].

2.3 SAMPLE PREPARATION AND ANALYTICAL METHOD

The sample preparation involved the fortification of 6 mL of urine with testosterone-d_3 and 17α-methyltestosterone at the final concentration of respectively 25 ng/mL and 125 ng/mL. The pH was adjusted to 7.5 by adding 2 mL of a 0.1 M phosphate buffer and some drops of NaOH 1 N. 100 µL of β-glucuronidase (corresponding to approximately 83 units) was subsequently added and the mixture was incubated at 58°C for 1 hour. Once the hydrolysis was completed, the mixture was cooled to room temperature and 2 mL of 0.1 M carbonate buffer (pH 9) were added. LLE was performed with 10 mL of TBME. Then, the sample was shaken in a multi-mixer for 10 minutes and subjected to centrifugation at 4000 rpm for 5 minutes. The organic phase was transferred into a vial and dried under nitrogen at 70°C. The dry residue was reacted with 50 µL of MSTFA/NH_4I/dithioerythritol (1.000:2:4 v/w/w) solution for 30 minutes at 70°C. A 1 µL aliquot of the resulting solution was injected into the GC-MS system working in the splitless mode. A typical chromatogram is reported in Figure 1 of the Supplemental Material.

The possible occurrence of urine sample degradation is associated with an increased amount of 5β-androstan-3,17-dione combined with a decrease of etiocholanolone concentration [29]. To assess the integrity of the samples, the same criterion adopted by the WADA [17] was used, e.g. a 5β-androstan-3,17-dione/Etio ratio higher than 0.1 was used as an indicator of significant microbial degradation and implied to discard the sample.

The specific gravity (SG) of each sample was measured, and the concentrations of all analytes were normalized for the dilution factor against the SG reference value of 1.020:

$$Conc_{correct} = Conc_{measured} \times (1.020-1)/(SG-1).$$

2.4 INSTRUMENTATION

Details about GC-MS instrumentation and operating conditions are reported in a previous study [18]. The MS was operated in the selected ion monitoring mode, and three diagnostic ions for each analyte were monitored with dwell times of 20-50 ms (Supplemental Material – Table 2).

The SG was measured with an Automatic Urinalysis System FUS-100/H800 (DIRUI Industrial, China) using a refractometric method.

2.5 METHOD VALIDATION

The calibration process was conducted with an optimized procedure, requiring the preparation of two replicates of the calibration curves for the 18 targeted steroids in three different days for a total of six calibration curves [30]. Several validation parameters could be determined from these data, including linearity range, selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ) trueness, intra and inter-assay precision, and repeatability. The data from each specific calibration curve were quantified using a calibration curve obtained in a different day, allowing to manage each set of data as independent. Therefore, 6 samples (from 6 different batches) per each calibration level were employed to estimate the previously cited validation parameters. Matrix effect, extraction recovery, and carry-over were calculated with separate experiments and regularly checked within daily working sessions. The linearity was evaluated within the concentration range of 2.0-500.0 ng/mL for the MIX A analytes (two independent calibration ranges were tested as follows: 2.0-125.0 ng/mL and 10.0-500.0 ng/mL – i.e. 2.0, 5.0, 10.0, 25.0, 50.0, 125.0, 250.0 and 500 ng/mL) and within the range of 100.0–5000.0 ng/mL for the MIX B (calibration ranges: 100.0-1500.0 ng/mL and 500.0-5000.0 ng/mL – i.e. 100.0, 200.0, 500.0, 1000.0, 1500.0, 2250.0, 3500.0 and 5000.0 ng/mL). The lowest concentration ranges typically correspond to the expected physiological levels [5]. The linear calibration parameters were evaluated using the least squares regression method; several significance tests were performed to evaluate linearity, including lack-of-fit tests, analysis of variance (ANOVA), Mandel's test, homo- vs. heteroscedasticity tests. Determination coefficient (R^2), relative standard deviation of the slope, normality of the

standardized residuals, and deviation from back-calculated concentrations were also evaluated. In-house spreadsheets, as well as package mvtnorm [31,32], and the routines developed by B. Desharnais et al. [30], working in the R environment, were used for this purpose. The LOD and LOQ were estimated by means of the Hubaux-Vos' algorithm [33].

To determine selectivity and specificity, the signal-to-noise ratio (S/N > 3) was measured on the selected ion chromatograms at the expected retention times for all the analytes of interest. The presence of interfering peaks around the retention time of the analytes was examined. Trueness, intra- and inter-assay precision were estimated as CV% and percent bias, respectively; satisfactory results were expected to lie within ±15%. Retention time repeatability was verified on 30 real urine samples together with blank water samples spiked at different concentration levels. Deviations below 1% from calibrators and controls were considered acceptable. Ion abundance repeatability was evaluated on the selected qualifying-ion chromatogram for each target analyte, with acceptance limit of ±20% with respect to the control. Carry-over effect was evaluated by injecting one distilled water extracts after the highest point of each calibration curve: if the signal-to-noise ratio was lower than 3 for in each ion chromatogram the carry-over effects was considered negligible.

The matrix effect was estimated at three concentration levels by comparing the experimental results obtained from blank urine samples (collected from a 5 months old female child) and blank deionized water samples, both spiked after the extraction step [34]. The matrix effect for each target analyte was expressed as the percentage ratio between the two measured concentrations. The extraction recovery was determined by comparing the experimental results from blank urine samples spiked respectively before and after the extraction step and was expressed as the percentage ratio between the two quantified concentrations.

2.6 SAMPLE COLLECTION

Single urine samples were collected from 241 Caucasian male volunteers, enrolled at the Department of Urology at the San Luigi Hospital of Orbassano (TO). They are subdivided as follow: (i) 212 healthy subjects (~88%), aged 18-80, who did not take any pharmaceutical drugs that could interfere with the USP; (ii) 29 (~12%) individuals affected by PCa, diagnosed by means of PSA > 4 and subsequent positive biopsy: symptoms of prostatic and urinary tract infection, results of TRUS biopsy, Gleason Score (from 2 to 10) and risk level (low, middle, high) were annotated. All the subjects belonging to the pathological class are older than 60 years.

All samples were collected in the morning. Detailed description of the cohort is provided in the section 3.2.

The choice of including only Caucasian subjects was to limit the UGT's polymorphisms and the corresponding variability factor. Biometric (weight and height) and anamnestic information were collected from all individuals. PCa patients submitted to pharmacological treatments were excluded from the study. All subjects provided a signed informed consent to donate urine (protocol number 0019267, approval of Ethical Committee of the San Luigi Hospital of Orbassano, Turin). The collected samples were frozen at −20°C and analyzed within the following 6 weeks. For each urine sample, 16 EAAS were detected, plus formestane and 5β-androstan-3,17-dione, viz. a marker of microbial degradation. Moreover, 5 steroid concentration ratios were calculated (i.e. T/E, A/T, A/Etio, 5α-adiol/5β-adiol and 5α-adiol/E). The microbial degradation did not occur for any sample (i.e. 5β-androstan-3,17-dione/Etio lower than 0.1), so the final dataset was constituted by 241 rows (subjects) and 22 columns (variables).

2.7 CHEMOMETRICS

A multivariate regression model was developed to study the correlation existing between the chronological age of healthy male individuals and their USP. The healthy subjects dataset used for the regression was constituted by a **X** matrix of dimensions 212 (i.e. number of the subjects) × 22

variables and a **Y** vector of the responses (i.e., the age of the tested subjects). A logarithmic transformation was applied to the **X** matrix in order to correct the non-normal distribution of the data into Gaussian distributions, manageable by the regression algorithms. The dataset was split into a training (80% of the dataset) and a test set, using the Kennard-Stone's algorithm [35,36]. Then, a variables selection was executed by means of genetic algorithms (GA) in the stepwise forward mode [37].

A Kernel-based Partial Least Squares (K-PLS) algorithm was used to calculate the regression model [38,39]. The model was submitted to double validation: during the procedure of model building, a cross-validation (CV) was performed (i.e. 20% of randomly chosen data were left out and used to test the prediction capability of the model); the most favourable model was chosen as the one with the lowest root-mean-square error in cross-validation (RMSECV). Then, an external validation was performed by predicting the response of the test set, and the root-mean-square error in prediction (RMSEP) was calculated.

The model was subsequently tested on the dataset of subjects affected by PCa (29 rows × 22 columns) and the new RMSEP was compared with the one obtained from the healthy subjects. In order to exclude the age-related bias from the data, a preliminary classification model was subsequently developed on a homogeneous sub-set of individuals (aged 60-80) with the purpose of evaluating the proficiency of USP to discriminate healthy individuals from PCa patients. The model was optimized in CV by venetian blind strategy with 5 elimination groups, and selecting the one with best RMSECV.

Matlab version R2013b was employed to perform the data pre-treatment, GA and the regression models. The PLS-Toolbox 8.0 was used for the classification purposes [40].

3. RESULTS AND DISCUSSION

3.1 VALIDATION OF THE ANALYTICAL METHOD

In the lower calibration range, the linear model provided the best calibration for 11 analytes and the quadratic model for the others. A constant $1/x^2$ weighting factor was used for all calibrations, after the results of calibration data-points proved to have heteroscedastic distribution. All the validation results and calibration equations are reported in the Supplemental Material (Tables 3-8), together with LOD and LOQ values. Retention time precision, selectivity and specificity proved to be satisfactory, and no interfering signals were detected at the retention times of the target analytes. Trueness, intra- and inter-assay precision results turned out adequate, as the percent bias and CV% values were lower than 15.0% at all the tested concentration levels.

Both extraction recovery and matrix effect turned out satisfactory (i.e. higher than 80% and lower than 120%) for all the target EAAS (Supplementary Tables 5-6). Lastly, absence of any carry-over effect was observed.

3.2 UNIVARIATE EXPLORATION OF THE HEALTHY POPULATION'S DATASET

The 212 healthy individuals were distributed over the investigated range of age as follows:

- age range 20 – 39 yrs: 53 individuals;
- age range 40 – 59 yrs: 59 individuals;
- over 60 yrs: 100 individuals

The number of variables composing the USP was reduced from 22 to 20, because the analytical signal for 7α-OH-testosterone and 4-OH-androstendione was always below the LOD. The general age-related trend shows a decrease of the urinary concentration for almost all steroids, as is evident in boxplots reported in Figure 1. More specifically, both A and Etio concentrations proved to decrease considerably with the aging. A significative decrease was also observed for 5α-Adiol and DHEA. For all the other steroids, a less-pronounced lowering of the median value is detected in the

high range of age. On the other hand, the urinary steroidal ratios do not show the same regular trend, with most median values nearly stable among the three ranges of age.

3.3 REGRESSION MODEL AND PLS-DISCRIMINANT ANALYSIS

A K-PLS regression algorithm was preferred to the traditional PLS-R approach, since in K-PLS input variables are projected onto a high-dimensional feature space to deduce the non-linear component from the data. Then, K-PLS can be considered as a conventional PLS algorithm operating in the feature space [38,39].

The regression model was built on the reduced (80%) USP dataset of healthy individuals (i.e., 169 subjects selected with the Kennard-Stone's algorithm to form the training set) after logarithmic (base-10) transformation and autoscaling.

The variable selection, performed using a hybrid GA (multiple GA runs followed by a forward stepwise inclusion of the most frequently picked predictors), resulted in the selection of 8 important variables, including T, 5α-adiol, 5β-adiol, A, DHEA, 7β-OH-DHEA, T/E, and A/Etio. The best performing regression model - based on Gaussian Kernel type, with σ equal to 2.69 - provided 4 latent variables, with R^2 equal to 0.75 and RMSECV of 8.7. The model is reported in Figure 2.A, featuring the subjects age predicted from USP data as a function of their real chronological age. The data distribution shows good correlation, yielding a prediction error lower than 10 years. The difference between the observed slope of the blue straight line (0.747) and the one of the red line representing exact correlation (1.000) provides another measure of the prediction error. The modelled correlation occurring between the multivariate USP of the individuals and their age was validated further, using the test set constituted by 43 USP from the healthy individuals previously discarded by the Kennard-Stone's algorithm. The data-points for each individual within the previous model is represented by red squares in Figure 2.B; a RMSEP of 13 was obtained.

A set of 29 individuals with prostatic malignancy was used to test the predictivity power of the regression model. In this case, the RMSEP was of 22 years, with bias and residuals values of −18.26 and −83.79 respectively (Figure 2.C). To investigate the reason of this lowered prediction power, the boxplots related to the steroids concentrations for the PCa population and the healthy individuals were built and reported in Figure 3. Within the eight variables selected for the regression of Figure2, an increased expression of the six EAAS is observed in the PCa population, while the two concentration ratios show similar distributions. In contrast, a significant difference in the urinary concentrations of Etio and 4-OH-testosterone is evident (Figure 3). Consequently, a new regression model was built, that excluded T/E and A/Etio ratios and included Etio and 4-OH-Testosterone concentrations. The new regression model performed worse than the original one in the age prediction, as is demonstrated by a R^2 value of 0.67 (instead of 0.75), although it showed a similar RMSEP value (11 years). On the other hand, a remarkable improvement in the separation of the two populations distribution (i.e., healthy vs. PCa-affected) is clearly observed in Figure 2.D.

Figure 4 illustrates the distribution of the error in prediction for both healthy and PCa-affected individuals in the first (Figure 2.A) and in second (Figure 2.B) regression model. For the healthy population the maximum of the curve that describes the error distribution remains almost unchanged, from 0 to 2 years, whereas the maximum of the error distribution for the PCa population increases from -22 (Figure 4.A) to -32 years (Figure 4.B), resulting in a noteworthy increase of the separation between the two populations.

Taking into account that PCa mostly occurs in individuals older than 60 years, a more homogeneous population of healthy individuals with age ranging between 60 and 80 years was extracted from the whole dataset, and a new K-PLS model was built (Supplementary Material, Figure 2), using the same 8 variables selected for the second regression model. The R^2 coefficient within this age interval drops to 0.3345, confirming that only a minor trend is present in the data distribution, allowing us not to consider the age-related bias in the USP interpretation.

The good separation of the two distributions reported in Figure 2.D induced us to investigate a multivariate approach to UPS data as a means to screen out PCa-affected individuals. A new dataset, composed by 83 healthy subjects older than 60 years and the 29 PCa-affected individuals, was split in training set (80%) and test set (20%). A classification model based on PLS discriminant analysis (PLS-DA) was developed using same variables selected for the second regression model. The PLS-DA model yielded excellent discrimination between the two groups, as is evident in Figure 5. The area under the curve (AUC) for the receiver operating characteristic (ROC) curves provides a value close to 0.99 in calibration and higher than 0.97 in CV, as is reported in Figure 6. The sensibility and specificity of the model for the class of pathological individuals are equal to 96% and 94%, respectively, confirming the potential of the PLS-DA application to UPS data for the screening of prostate carcinoma.

4. CONCLUSIONS

The biosynthesis of EAAS is largely dependent on age, which - for male individuals - is reflected in a progressive modification of their urinary EAAS profile. As these profiles are considered in their multivariate expression, a strong linear correlation was found with the chronological age. Significant deviations from this relationship observed between chronological age and the "physiological age" derived from urinary EAAS profile may be symptomatic of a pathological condition, as we demonstrated for prostatic carcinoma. As a matter of fact, the regression model built from healthy subjects does not hold for individuals affected by PCa and this phenomenon can be attributed to the alteration of the EAAS biosynthesis caused by the prostatic pathology, which creeps over the effect of ageing and the other sources of variability.

Restricting the data evaluation to the typical late age of PCa onset, the chronological evolution of the multivariate EAAS profile becomes negligible, enhancing the possibility to distinguish the pathological from healthy conditions by means of classification models. Indeed, supporting

evidence of the diagnostic potential of urinary EAAS profiles was obtained by applying the PLS-DA classification algorithm to sub-sets of profiles obtained from over-60 healthy and pathological male subjects. Further improvements of the screening and diagnostic power of multivariate strategies to EAAS profiles interpretation could arise from larger sets of free and conjugated targeted EAAS and particularly from various untargeted analysis, allowed by both GC-MS and LC-HRMS methods. Indeed, these preliminary results encourage us to undertake a systematic study on the screening potential of multivariate discriminant analysis applied to a wide set of urinary androgenic steroids and addressed to the identification of PCa and its distinction from both prostatic hyperplasia and non-pathological conditions. This analysis may support the traditional screening procedure, involving digital rectal examination, PSA determination and TRUS biopsy.

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Table 1: Composition and concentration of analytes and internal standards in the working solutions.

Solution	Composition	Concentration (µg/mL)
MIX A	T, E, 5α-adiol, 5β-adiol, DHEA, 5β-androstan-3,17-dione, 4-androsten-3,17-dione, 4,6-androsten-3,17-dione, 7β-DHEA, 7α-OH-testosterone, 4α-OH-testosterone, 16α-OH-androsten-3,17-dione, 5-androsten-3,17-diol, Δ6-testosterone, formestane, DHT	10
MIX B	A, Etio	200
MIX C	testosterone glucuronide, 5α-dihydrotestosterone glucuronide, dehydroepiandrosterone glucuronide, epitestosterone glucuronide	15
ISTD1	testosterone-d3	10
ISTD2	17-methyltestosterone	10

FIGURE LEGENDS

Figure 1: Box-plots reporting the urinary steroid concentrations and their ratios for three age ranges: 20-39 y, 40-59 y, and over 60 y, respectively.

Figure 2: (A) K-PLS model over the reference population; (B) prediction of the test set composed by healthy individuals; (C) prediction of the test set constituted by subjects suffering from PCa; (D) prediction of the test set constituted by subjects suffering from PCa using the new selected variables.

Figure 3: Box-plots reporting the eight biomarkers' values selected in the regression of Figure 2A, plus 4-OH-Testosterone and Etiocholanolone, limited to the "over 60" age-range and distinguished between healthy and PCa-affected populations.

Figure 4: Histograms reporting the distributions of the errors in prediction for healthy and PCa populations for the first (A) and the second (B) regression models, reported in Figures 2C and 2D.

Figure 5: PLS-DA model separating the healthy (blue dots) and PCa (green squares) populations in the age range over 60 years.

Figure 6: ROC curves (left) and responses (right), both estimated and cross-validated, derived from the PLS-DA model reported in Figure 5.

Figure 1

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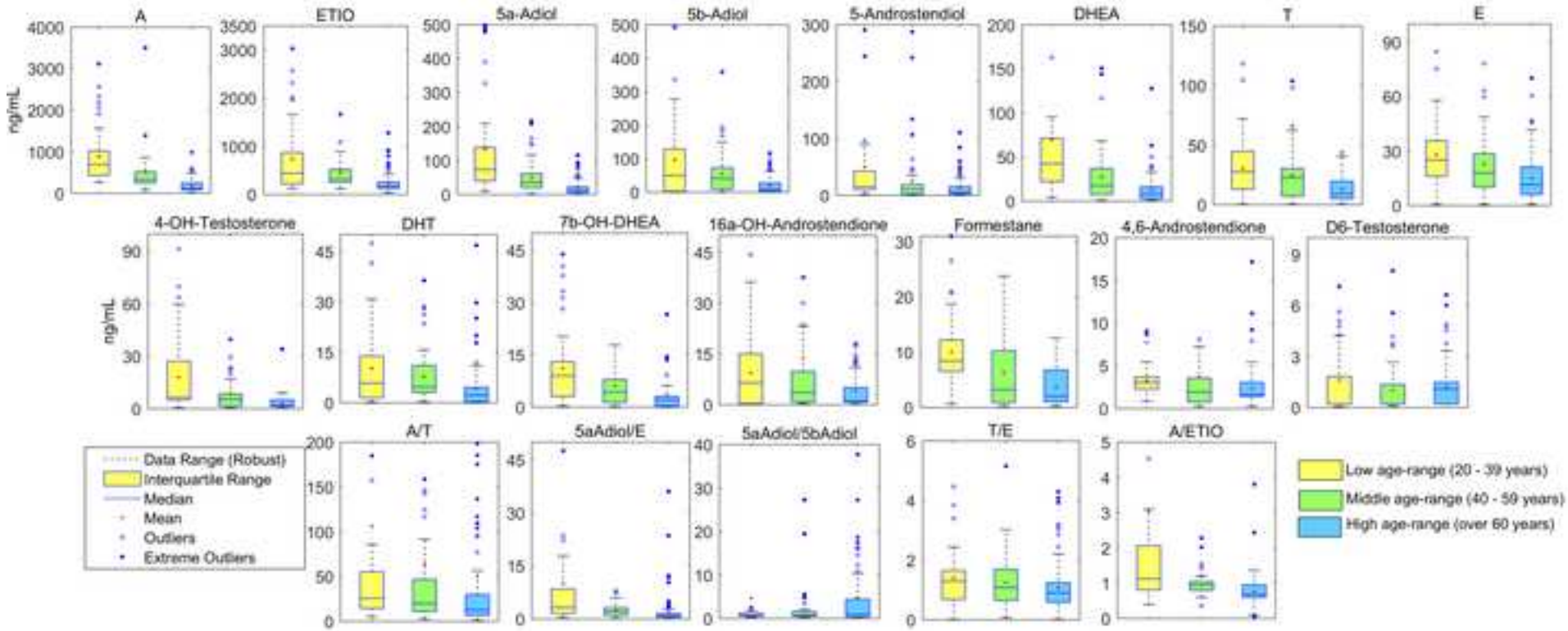

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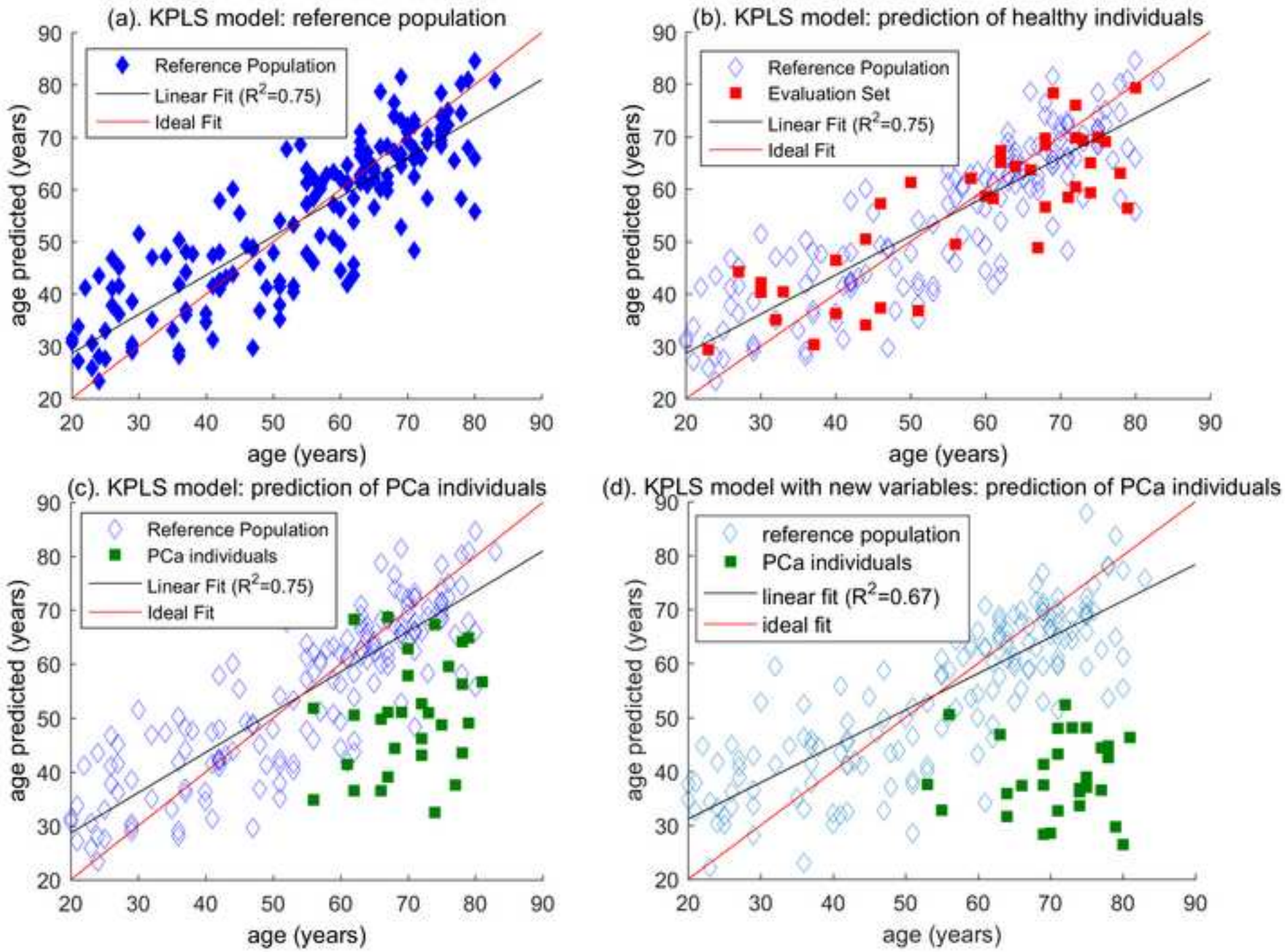

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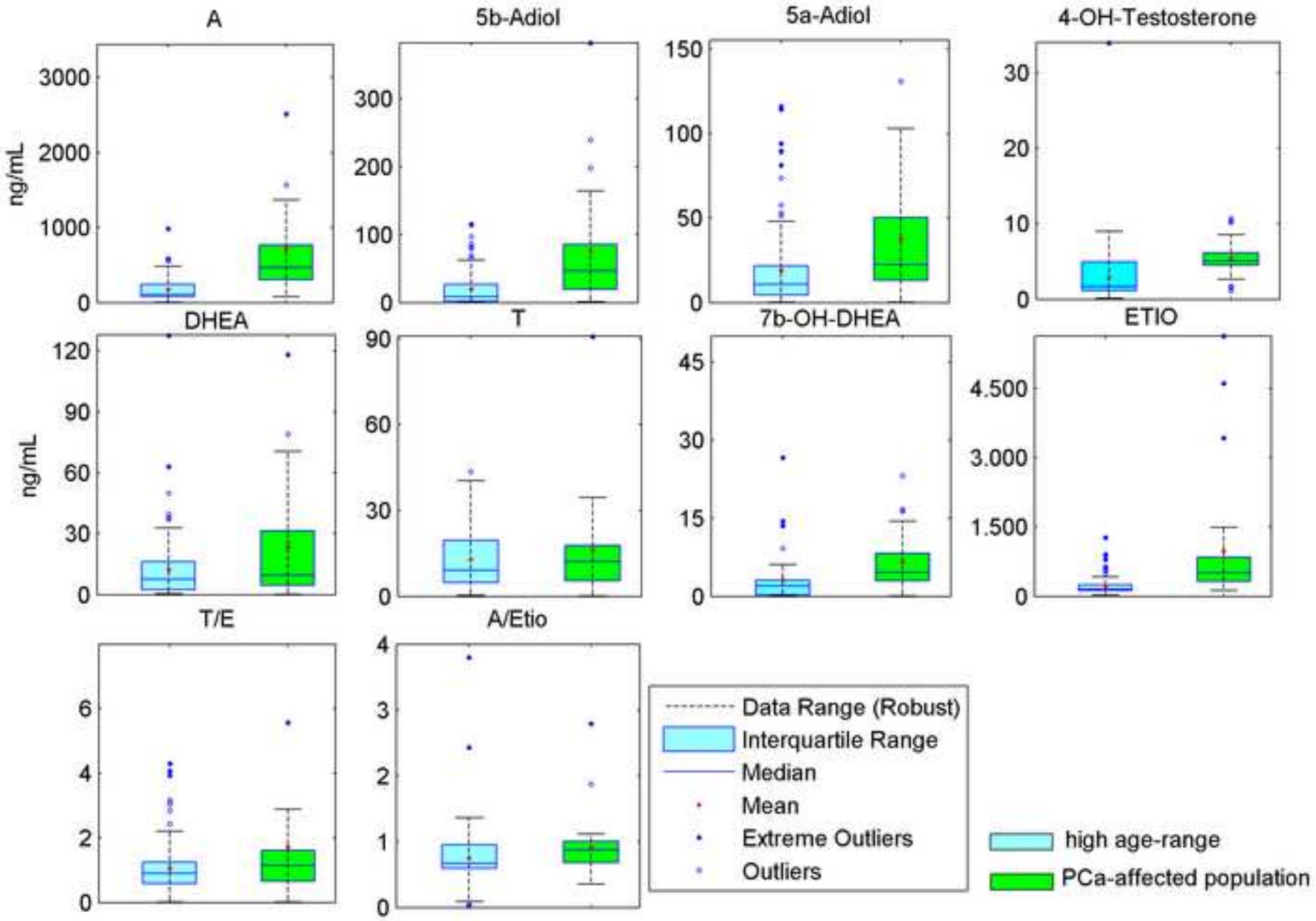

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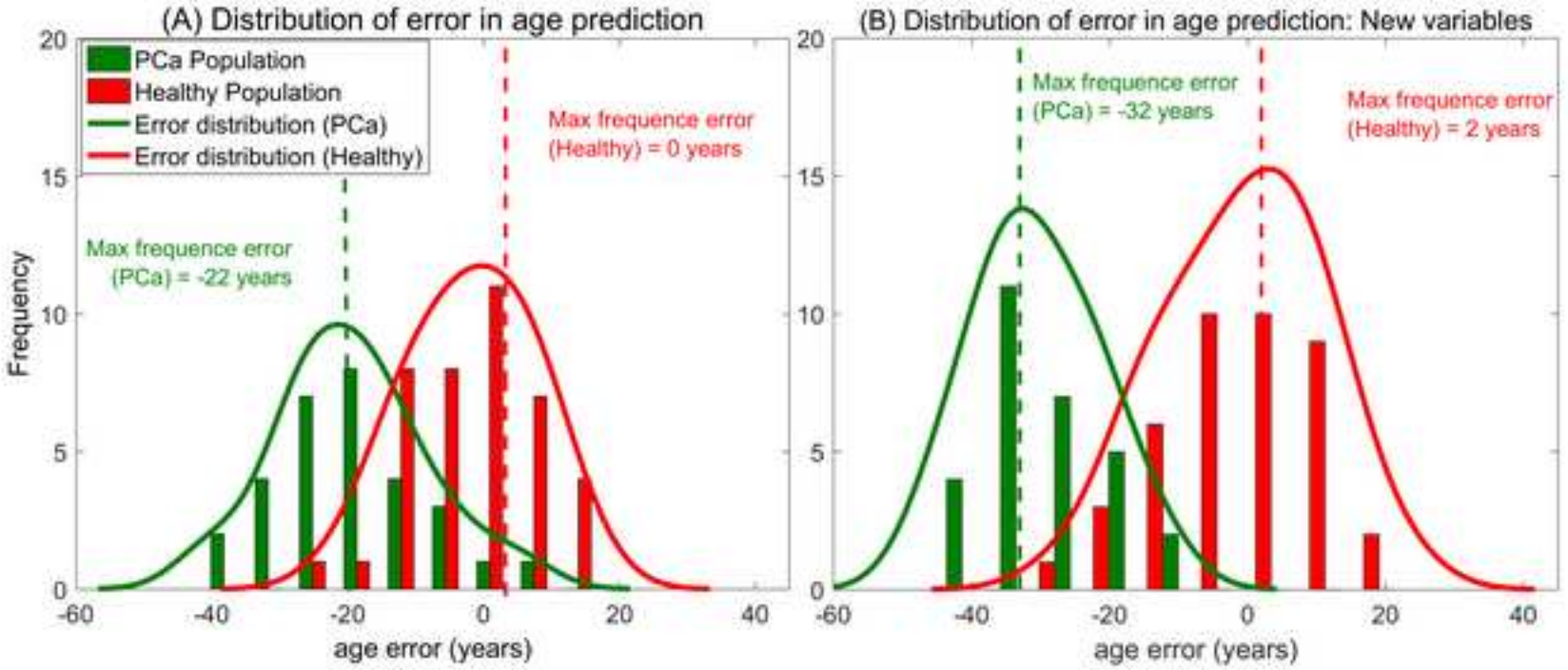

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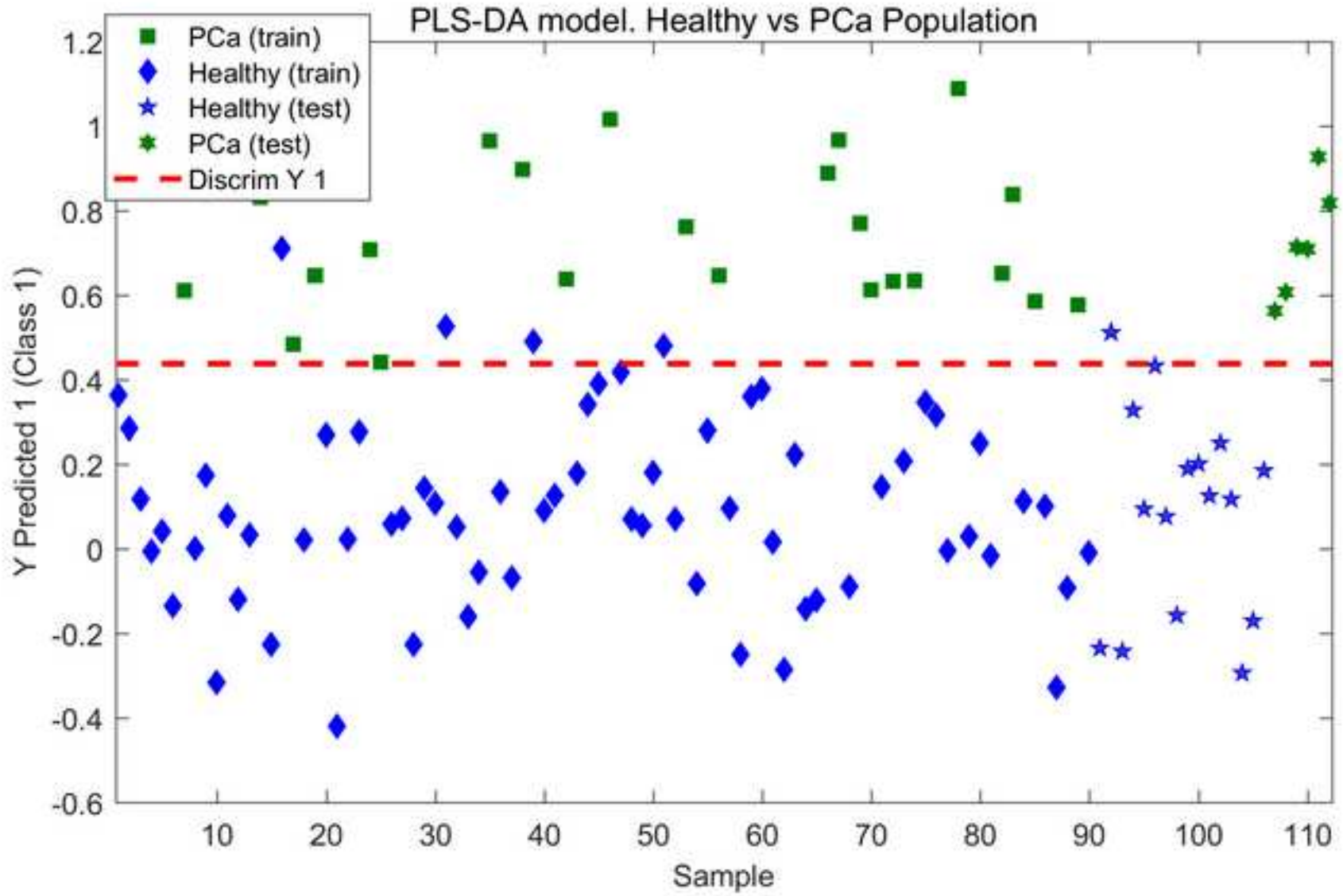

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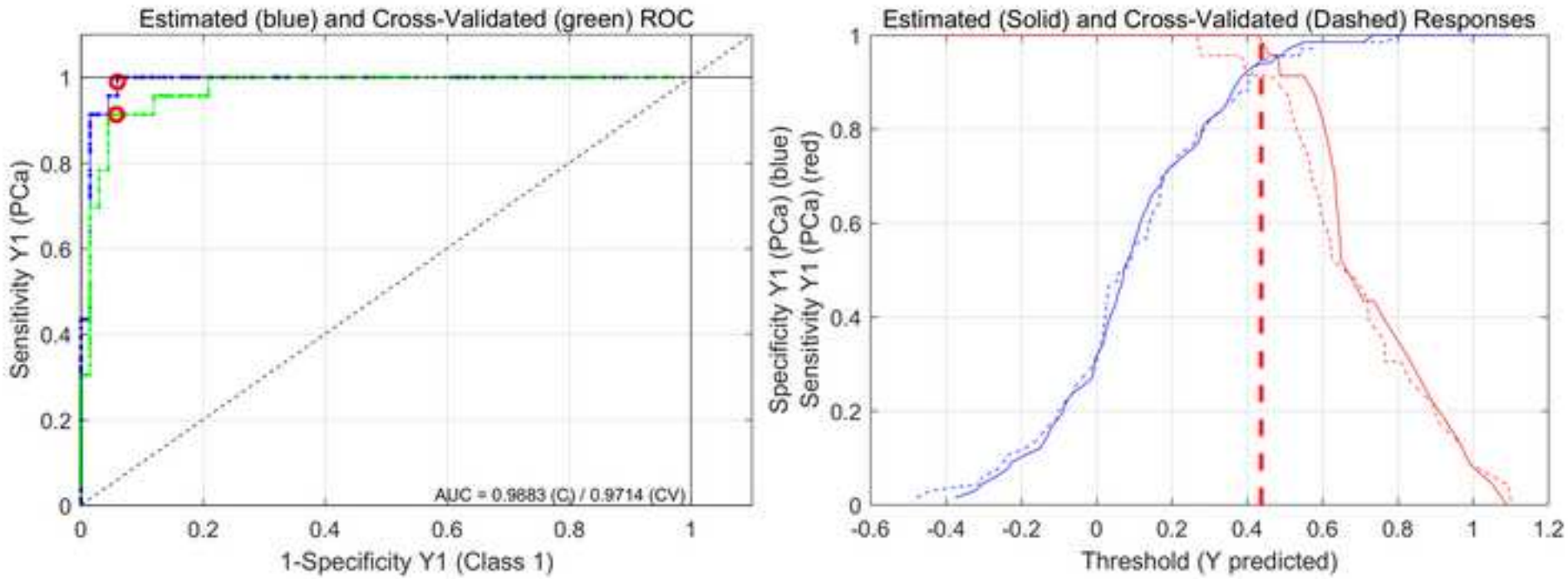

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